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(57) Abstract

A method for screening a chemical compound for hepatotoxicity or choleostatic potential, hepatic extraction potential, hepatic discharge into the bile, metabolite production, or the ability to alter hepatic cell metabolism, using a primary hepatic cell culture with a functional bile canalicular network, is disclosed.

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TITLE OF THE INVENTION
HEPATIC MODEL

FIELD OF THE INVENTION

This invention pertains to both a novel and useful method for screening a chemical compound for hepatotoxicity or choleostatic potential, hepatic extraction potential, hepatic discharge into the bile, metabolite production, or the ability to alter hepatic cell metabolism, using a primary hepatic cell culture with a functional bile canalicular network. The use of this novel method results in less of a dependence on whole animal toxicity and drug discovery studies and reduces the total number of animals required for chemical testing. In addition, information obtained from this screening procedure is devoid of interference from reactions which may occur with other organs and tissue when whole animal studies are conducted.

BACKGROUND OF THE INVENTION

Scientific, financial and ethical considerations regarding the use of whole animal studies for screening new drug candidates for hepatotoxicity or choleostatic potential, hepatic extraction potential, hepatic discharge, metabolite production and the ability of a chemical compound to alter hepatic cell metabolism have led to a search for an in vitro/ex vivo system which accurately reflects the in vivo behaviors of chemical compounds.

The influence of extracellular matrix geometry on hepatocyte function has been reported using a sandwich configuration by Dunn et al. (See J.C.Y. Dunn et al., The FASEB Journal, Vol 3, 174 - 177 (1992); The Journal of Cell Biology, Vol 116, 1043 - 1053 (1992); and Biotechnol. Prog. Vol 7, 237-245 (1991).) In this work, adult rat hepatocytes cultured in a collagen sandwich system maintained normal morphology and a physiologic rate of secretion of albumin, transferrin, fibrinogen, bile acids and urea for at least 42 days. These cells were shown to maintain the level of albumin mRNA similar to that found in the normal liver for this same time period.

The subject matter of this invention has demonstrated that hepatic cell cultures are useful in determining the effect of chemical compounds on hepatic cells and the effect of hepatic cells on the compounds. Rat and porcine hepatic cell cultures were grown on a hard or soft substrate, that is, ungelled or gelled collagen, and subsequently overlaid with a collagen gel. These cells initiated uniform formation of bile canaliculi throughout the entire culture. Typically, 24 hours after overlaying the collagen gel, the hepatic cell cultures formed are characterized by a nearly complete and continuous bile canaliculi network. Morphological analysis of these "sandwiched" cell cultures by electron microscopy confirmed that channels had formed between most neighboring cells and that these channels possessed many of the characteristics of native bile canaliculi, such as junctional complexes, microvilli and a terminal actin web subjacent to the apical membrane. These canaliculi are also functional at concentrating xenobiotic agents presented to the hepatocyte culture. It has therefore been demonstrated in this invention that hepatic cells cultured in this manner can be used to screen a wide variety of chemical compounds with only limited sacrifice of laboratory animals.

SUMMARY OF THE INVENTION

A method is presented for the in vitro screening of a chemical compound for hepatotoxicity or choleostatic potential, hepatic extraction potential, hepatic discharge, metabolite production or the ability of a chemical compound to alter hepatic cell metabolism, using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 to 2 times 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 fM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 1 minute to about 2 weeks;

(j) assessing morphologic or structural changes in the integrity of the canalicular network, cell polarity or cytoskeletal structure; quantifying the amount of the chemical compound contained within the hepatic cells or remaining in the culture solution; quantifying the chemical compound secreted into the bile contained within the canalicular network; or measuring the extent of alteration of hepatic cell metabolism.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have found a novel chemical compound screening procedure for the in vitro assessment of hepatotoxicity or choleostatic potential, hepatic extraction potential, hepatic discharge, metabolite production or the ability of a chemical compound to alter hepatic cell metabolism, using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 to 2 times 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 fM to about 50 mM of the chemical compound in a solvent

which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 1 minute to about 2 weeks;

(j) assessing morphologic or structural changes in the integrity of the canalicular network, cell polarity or cytoskeletal structure; quantifying the amount of the chemical compound contained within the hepatic cells or remaining in the culture solution; quantifying the chemical compound secreted into the bile contained within the canalicular network; or measuring the extent of alteration of hepatic cell metabolism.

In accordance with the practice of this invention, it has been found that primary hepatic cells grown on a hard or soft collagen substrate and subsequently overlaid with a collagen gel initiated uniform formation of bile canaliculi throughout the entire culture which can be used in the screening of chemical compounds. Typically, 24 hours after overlay, hepatic cell cultures formed an anastomosing bile canaliculi network interconnecting a plurality of cells. Morphologic analysis of these "sandwiched" hepatic cell cultures confirmed that channels had formed between most neighboring cells and that these channels possessed many of the characteristics of native bile canaliculi, such as, junctional complexes, microvilli, and a terminal actin web subjacent to the apical membrane.

Immunostaining "sandwiched" hepatic cell cultures with antibodies against apical membrane markers (aminopeptidase N, dipeptidyl peptidase IV) showed intense fluorescence staining at the plasma membrane regions associated with the channel structure. In addition, double fluorescent labeling of hepatic cell cultures for actin microfilaments and microtubules before and after channel formation illustrated that an extreme reorganization of the cellular actin and tubulin occurs during channel formation. The actin staining pattern changed from a diffuse distribution of stress fibers to an intense, peripheral staining subjacent to the channel membranes. This rearrangement of actin staining was consistent with the formation of a

pericanalicular actin web around newly formed channels observed in electron micrographs. Tubulin staining showed arrays of bundles of microtubules attaching to and radiating from the leading edges of newly forming canaliculi.

The hepatic cell culture is produced by plating primary hepatocytes on tissue culture dishes coated with gelled or ungelled collagen basement matrices, allowing the cells to spread out and make intercellular contacts, then overlaying and gelling extracellular matrix on top of the cultures. Tissue culture dishes are coated collagen at least 1 day prior to preparing the hepatocytes. To obtain a hard, ungelled growth substrate, 200-250 µl of 3 mg/ml Vitrogen® (or other collagen solution) is added to each 100 mM tissue culture dish and spread evenly with a teflon policeman. Coated dishes are placed at 37°C overnight then 5 ml fresh tissue culture media is added to neutralize the collagen. To obtain a gelled basement matrix, neutralized Vitrogen® is prepared (8:1:1 Vitrogen®, 10X Dulbecco's modified Eagles medium, 0.1 N NaOH) and spread onto petri dishes as described above. The plates are placed at 37°c for 30-40 min to allow the collagen gel to form, then fresh media is added to the dishes which are stored at 37°C. Just before use, the media is aspirated from the precoated culture dishes.

Hepatocytes are isolated using standard collagenase perfusion methods (Selgen, P.O. METHODS BIOL., 1976 13, 29-83. Livers are perfused with calcium-free buffer for 8-9 min followed by buffer containing calcium and collagenase (0.3 mg/ml) for ~10-12 min. The liver is then cut open and released liver cells are separated from undigested tissue with a sterile nylon mesh. The released cells are then divided into two 50-ml centrifuge tubes and washed 1X with Dulbecco's modified Eagles medium containing 5% fetal calf serum, without hormonal supplements. The cell pellets are resuspended in a 1:1 mixture of 90% isotonic Percoll and media and centrifuged for 5 min at 350 X g . The pellets containing viable hepatocytes are resuspended in fresh media, washed one time, and the hepatocytes are added to precoated dishes at a density of 9×10^6 cells/dish and incubated at 37°C in a 5% CO_2 incubator. After 2-3 hours, media is replaced with fresh,

warm media containing 0.4 μg/ml dexamethasone, 4 μg/ml insulin, and 20 ng/ml EGF. Media was subsequently replaced on a daily basis with fresh, warm media containing hormonal supplements. Twenty-four to forty-eight hours after plating the cultures are overlaid with a collagen gel by aspirating off the culture media and adding 1 ml of neutralized collagen (8:1:1 Vitrogen: 10X Dulbecco's modified Eagles medium: 0.1 N NaOH). Cultures are then incubated at 37μC for 30-40 min to allow the matrix to gel, after which fresh media is added back to the dish. The formation of bile canaliculi is then monitored with phase contrast microscopy with an inverted microscope.

In one embodiment of the invention, hepatotoxicity or choleostatic potential of a chemical compound can be assessed by adding from about 1 μl to about 5 ml of a solution containing from about 1 fM to about 1 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2 for from about 5 minutes to about 96 hours. The hepatic cell culture is then examined to determine the extent of any toxic effect on the hepatic cells produced by the chemical compound.

This method of screening chemical compounds for hepatic cell toxicity is particularly convenient since the formation of well defined canaliculi allows direct observations to be made of morphological changes to the canaliculi.

In a second embodiment, a method for screening a chemical compound for hepatic extraction potential using this "sandwiched" hepatic cell culture is accomplished by adding from about 1 μl to about 5 ml of a solution containing from about 1 pM to about 50 mM of the chemical compound to be screened to the culture and incubating the hepatic cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2 for from about 1 minute to about 4 hours. The amount of the chemical compound contained within the hepatic cells is determined by removing the drug containing buffer and washing the cells with an isotonic salt solution (ie. phosphate buffered salt solution

or Hank's balanced salt solution), lysing the cells with detergent solutions (ie. sodium dodecyl sulfate) and determining the amount of drug in the lysate and the original incubation solution using analytical procedures such as scintillation counting, HPLC, absorbance or fluorescence quantitation. Alternatively in the case of fluorescent chemical compounds direct quantitation of the chemical compound can be assessed by direct determination of cell associated fluorescence.

This method of screening chemical compounds for hepatic cell toxicity is particularly convenient when the compound is transported into the hepatic cells using active carrier systems such as Type I cationic, Type II cationic, anionic, bile salts and asialoglycoprotein receptors, or when the compound is passively absorbed into the hepatic cells since they represent the normal for extraction of chemical compounds by the liver.

In a third embodiment of this invention, screening of metabolite production from the interaction of the hepatic cell culture and a chemical compound is accomplished. In this screening procedure, from about 1 µl to about 5 ml of a solution containing from about 1 pM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture is added to the culture plate. The culture is then incubated at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2 for from about 30 minutes to about 2 weeks. At discrete times, samples of the incubation media can be taken and once incubation is complete, loss of the parent compound and production of new metabolites can be determined using chemical analysis techniques such as HPLC, TLC, mass spectroscopy. Similarly cell associated parent compound and metabolites can be determined from detergent cell lysates.

In a forth embodiment of this invention, the potential of a chemical compound to alter hepatic cell metabolism using this screening process is also within the scope of Applicants' invention. In this process, from about 1 µl to about 5 ml of a solution containing from about 1 pM to about 50 mM of the chemical compound in a compatible solvent is added to the hepatic cell culture. Substrate for a metabolic

pathway is added and the culture is incubated at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2 for from about 30 minutes to about 2 weeks. The metabolism of the hepatic cells is determined by identifying and quantifying the formation of products from the substrate in the presence and absence of the chemical compound.

A fifth embodiment of this invention relies on the fact that hepatocytes grown in this collagen sandwich configuration have been shown to be competent at xenobiotic excretion into the canaliculi. The potential for hepatic discharge of a chemical compound into bile may be determined using this screening process. From about 1 µl to about 5 ml of a solution containing from about 1 pM to about 50 mM of a chemical compound to be screened, in a solvent which is compatible with the hepatic cell culture, is added to the hepatic cell culture. The culture is then incubated at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2 for from about 1 minute to about 4 hours. At this time the cells are washed using phosphate buffered saline and then the canaliculi are disrupted with EDTA. That is, EDTA disrupts the tight junctions which seal the canaliculi without breaking cell membranes. This technique allowed for the measurement of material which was secreted into the canalicular network without interference from the material which had been incorporated into the cell. When 5 µl of 1 mg/ml carboxyfluorescein diacetate or 5 µl of 1 mg/ml rhodamine B were introduced into the medium, these compounds were observed by fluorescence microscopy to be selectively secreted and concentrated into canalicular network of "sandwiched" hepatocytes. Carboxyfluorescein fluorescence was released by treatment with ethylenediaminetetraacetic acid (EDTA) which is consistent with the formation of macrodomains bounded by tight junctions.

This method is also applicable to the screening of many chemical compounds simultaneously. Any number of compounds which can be solubilized by the compatible solvent may be delivered to the hepatic cell culture simultaneously. In the event that hepatotoxicity is observed, each of these compounds may then be individually screened to

determine which compound or groups of compounds produced the effect.

By "screening" is meant the determination of potential for specified activity for a series or mixture of compounds.

By "chemical compound" is meant any chemical agent or mixture of chemical agents with potential toxic or therapeutic properties.

By "continuous" is meant that canaliculi form a multicellular network of channels throughout the cell culture which integrate a plurality of cells within the culture and are contiguous to many cells as distinct from isolated cell couplets.

By "hepatotoxicity" is meant an activity which results in deleterious effects on normal liver function or diminishes the viability of liver cells.

By "choleostatic potential" is meant the propensity to decrease normal biliary output.

By "primary hepatic cell culture" is meant normal liver cells maintained in vitro.

By "functional bile canalicular network" is meant anastomosing interconnected tubular canals between hepatocytes which maintain the capability for directional secretion of chemical compounds.

By "collagenase perfusion" is meant a technique for separating and isolating hepatocytes wherein in situ perfusion of the liver with a collagenase solution results in enzymatic disruption of intercellular contacts and cell/basement membrane contacts.

By "hepatocyte culture media" is meant a maintenance solution containing nutrients and growth factors necessary for supporting hepatocyte viability, for example, Dulbecco's modified medium with 5% fetal calf serum, nonessential amino acids, glutamine, antibiotics, antimycotics supplemented with 0.4 μg/ml dexamethasone, 4 ug/ml insulin, and 2 ng/ml epidermal growth factor.

By "Percoll solution" is meant a mixture of colloidal polyvinylpyrrolidone coated silica used for centrifugal separation of cells and cellular components on the basis of density.

By "culture dish precoated with collagen" is meant a plastic well or plate in which a collagen gel has been adsorbed onto the surface or ungelled collagen has been dried onto the surface.

By "overlaying about 1 ml of cold neutralized collagen" is meant the process of depositing a solution of collagen on the top of cells previously adhered to a culture dish or well.

By " intercellular contact" is meant adhesions between neighboring cells.

By "canalicular network is developed" is meant a meshwork of continuous canaliculi has formed which interconnects multiple cells.

By "solvent which is compatible with the hepatic cell culture" or "compatible solvent" is meant a solution which, over the normal course of experimental procedures and hepatic cell maintenance, does not elicit adverse biochemical or morphologic changes in cultured hepatic cells.

By "morphologic or structural changes in the integrity of the canalicular network" is meant alterations to the physical appearance of the canaliculi and/or redistribution of membrane components or cytoskeletal components, i.e. actin or tubulin.

By "cell polarity" is meant non-uniform distribution of cellular and membrane components which confer a unique sidedness to a cell, i.e. a side which faces the canaliculi and a side which faces sinusoidal spaces.

By "hepatic extraction potential" is meant the tendency for a chemical compound to be absorbed into the liver.

By "the potential for hepatic discharge of a chemical compound into bile" is meant the tendency for a chemical compound to be secreted into bile canaliculi.

By "screening of metabolite production from a chemical compound" is meant the determination of the potential of a chemical compound to be chemically modified by enzymatic pathways in the liver.

By "the potential of a chemical compound to alter hepatic cell metabolism" is meant the ability of a chemical compound to interact with a biochemical pathway in the liver producing quantitative or qualitative changes in a product.

By "measuring the extent of alteration of hepatic cell metabolism" is meant using analytical techniques, such as radiolabeled- or fluorescent substrates or chemical analysis, to determine quantitative or qualitative changes in product formation from biochemical pathways.

The chemical compound used in this screen can be quantified using any standard analytical technique. Standard analytical techniques include but are not limited to the use of high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), electrophoresis, optical absorption, fluorescence quantitation, quantitation of radioactive isotopes.

Assessment of morphologic or structural changes in the integrity of the canalicular network, cell polarity or cytoskeletal structure is accomplished using light microscopy, fluorescence microscopy, electron microscopy, chemical modification of proteins, antibody labelling.

EXAMPLES

EXAMPLE 1

Effect of collagen overlay on rat hepatocytes

Experiments were performed in which neutralized bovine dermal collagen (Vitrogen®) was allowed to gel on the top surface of primary rat hepatocytes grown on tissue culture plates coated with dried ungelled collagen or with gelled collagen. After the overlaid collagen matrix gelled, tissue culture media was added to the dish and the cells were incubated at 37°C in a 95% air 5% CO_2 humidified incubator. Phase contrast microscopy of the cells 1 day later showed that the cells had developed an elaborate network of canalicular channels which extended across a plurality of cells which appeared as refractile channels which circumscribed the cells. These channels were confirmed to be bile

canaliculi by electron microscopy and fluorescence microscopy. Electron microscopy of the overlaid hepatocytes showed microvilli containing channels between neighboring cells which are bound on each side by tight junctions and have a dense actin network subjacent to the canalicular membranes. Using fluorescent-labeled antibodies, fluorescence microscopy confirmed the presence of the apical markers aminopeptidase N and Dipeptidyl peptidase in the canalicular channels similar to observations in intact liver. Similarly, fluorescent-conjugates of phalloidin demonstrated that actin in the hepatocytes is highly localized to the pericanalicular region similar to in intact liver.

The morphology of collagen overlaid cells grown on gelled collagen basement membrane was found to be better than cells grown on dried collagen as assessed by electron microscopy. Phase contrast microscopy showed that rat tail collagen, bovine tendon collagen, and bovine dermal collagen were all effective matrixes for the basement membrane and for the overlaid matrix, and that extracellular matrix from Engelbreth-Holm-Swarm mouse tumors (Matrigel™) was also effective as an overlay matrix.

EXAMPLE 2

Effect of collagen overlay on porcine hepatocytes

Experiments were performed in which neutralized collagen (Vitrogen®) was allowed to gel on the top surface of primary porcine hepatocytes grown on tissue culture plates coated with dried ungelled collagen or with gelled collagen. After the overlaid collagen matrix gelled, tissue culture media was added to the dish and the cells were incubated at 37°C in a 95% air 5% CO_2 humidified incubator. Phase contrast microscopy of the cells, 1 day later, showed that the cells had developed an elaborate network of canalicular channels which extended across a plurality of cells similar to observations with rat liver hepatocytes.

EXAMPLE 3

Collagen overlaid hepatocytes concentrate xenobiotics into their bile canaliculi

Experiments were performed to demonstrate the ability of collagen sandwiched hepatocytes to transport dicarboxyfluorescein from the interior of cells to the canaliculi and concentrate the compound in the canaliculi. Five days after collagen overlaying, the cultures were incubated at 37°C in Hanks balanced salt solution containing 1-10 µg/ml dicarboxyfluorescein diacetate to allow uptake of dicarboxyfluorescein diacetate and conversion of the probe to dicarboxyfluorescein. After five to fifteen minutes residual dicarboxyfluorescein diacetate was removed and the cells were washed 3-4 times with Hank's balanced salt solution. Uptake of the dicarboxyfluorescein was confirmed by fluorescence microscopy and was localized to the cell interior as well as some accumulation in the bile canaliculi. After 15-30 minutes of further incubation at 37°C comparisons of phase contrast and fluorescence microscopy showed the dicarboxyfluorescein to be localized almost exclusively in the bile canaliculi. Using 2mM EDTA the dicarboxyfluorescein concentrated in the canaliculi is released after disruption of the tight junctions which seal the canaliculi.

EXAMPLE 4

Passive absorption and secretion into bile canaliculi by collagen sandwiched hepatocytes

Experiments were performed to demonstrate that Rhodamine B which is passively absorbed by the liver and secreted into the bile is taken up and secreted into the bile canaliculi by collagen sandwiched hepatocytes. Five days after collagen overlaying, the cultures were incubated at 37°C in Hanks balanced salt solution containing 1 µg/ml rhodamine B. After five minutes residual rhodamine B was removed and the cells were washed 3-4 times with Hank's balanced salt solution. After 5-20 minutes further incubation at 37°C

comparisons of phase contrast and fluorescence microscopy showed the rhodamine B to be highly concentrated in the canaliculi and less concentrated diffusely distributed in the cell interior.

EXAMPLE 5

Active uptake of bile salts by collagen sandwiched hepatocytes

Experiments were performed to demonstrate active uptake of bile acids by Collagen sandwiched hepatocytes. Twenty-four hours after overlaying collagen, hepatocytes were rinsed with Hanks balanced salt solution and incubated with 1mM taurocholate with 0.1 μCi ^{14}°C-taurocholate at 37°C or at 4°C. At set time points the taurocholate containing buffer was removed and the culture plates were washed with Hank's balanced salt solution. The hepatocytes were then lysed with detergent and the amount of taurocholate taken up by the hepatocytes was determined based on the amount of radioactive taurocholate present in the lysate. The active uptake of taurocholate was distinguished from the passive uptake and adsorption based on the difference between uptake at 37°C and 4°C. Uptake experiments were also performed on unsandwiched hepatocytes at comparable cell density and showed that the collagen overlaid hepatocytes were better at taurocholate uptake (see Table 1).

TABLE I. TAUROCHOLATE UPTAKE

	Taurocholate Uptake (nMoles/ dish)		
Time:	0 min	2 min	15 min
Collagen Sandwiched	0	27	40
Unsandwiched	0	12	25

WHAT IS CLAIMED IS:

1. A method for screening a chemical compound for hepatotoxicity or choleostatic potential using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 - 2 x 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 fM to about 1 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and

incubating at the conditions of step (d) for about 5 minutes to about 96 hours;

(j) assessing morphologic or structural changes in the integrity of the canalicular network, cell polarity or cytoskeletal structure.

2. A method for screening a chemical compound for hepatic extraction potential using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 - 2 x 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1

pM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 1 minutes to about 4 hours;

(j) quantifying the amount of the chemical compound contained within the hepatic cells or remaining in the solution of step (i).

3. A method for screening the potential for hepatic discharge of a chemical compound into bile using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 - 2 x 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 pM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 1 minutes to about 4 hours;

(j) quantifying the chemical compound secreted into the bile contained within the canalicular network.

4. A method for screening of metabolite production from a chemical compound using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 - 2 x 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 pM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 30 minutes to about 2 weeks

(j) quantifying the loss of chemical compound or production of metabolites.

5. A method for screening the potential of a chemical compound to alter hepatic cell metabolism, using a primary hepatic cell culture with a functional bile canalicular network, the process comprising the steps of:

(a) isolating hepatic cells by collagenase perfusion;

(b) washing the isolated cells with hepatocyte culture media, suspending the cells in about a 50:50 mixture of the hepatocyte culture media and about 90% isotonic Percoll solution, and centrifuging at about 350 times gravity for about 5 minutes;

(c) adjusting the cell concentration to about 1 - 2 x 10^6 cells/ml of hepatocyte culture media and dispensing about 6 ml of cell suspension to a culture dish precoated with collagen;

(d) incubating the dish containing the cells at about 37°C, about 95% relative humidity and an atmosphere of about 5% CO_2;

(e) changing and replacing the hepatocyte culture media after about 2 hours to remove any unattached cells;

(f) overlaying about 1 ml of cold neutralized collagen solution to the dish containing the hepatic cells after the cells have spread and established intercellular contact, and

reincubating at the conditions of step (d) to allow the added neutralized collagen to gel;

(g) adding to the hepatic cells about an additional 5 ml of hepatocyte culture media;

(h) incubating at the conditions of step (d) until the canalicular network is developed as observed by light microscopy;

(i) adding from about 1 µl to about 5 ml of a solution to the hepatic cell culture, the solution containing from about 1 pM to about 50 mM of the chemical compound in a solvent which is compatible with the hepatic cell culture, and incubating at the conditions of step (d) for about 30 minutes to about 2 weeks

(j) measuring the extent of alteration of hepatic cell metabolism.

6. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the chemical compound is transported into hepatic cells using active carrier systems selected from the group consisting of Type I cationic, Type II cationic, anionic, bile salts, and asialoglycoprotein receptors.

7. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the chemical compound is passively absorbed into the hepatic cells.

8. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the hepatic cell culture is maintained between a basement and overlaid gelled extracellular matrix.

9. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the functional bile canaliculi form an anastomosing network interconnecting a plurality of cells.

10. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the hepatic cells are obtained from rat and porcine liver.

11. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the collagen is gelled on the culture dish prior to the addition of the cell suspension.

12. The method of screening a chemical compound as in claims 1, 2, 3, 4 or 5 wherein the collagen stock solution is equal to or greater than 3 mg/ml.

13. The method of screening a chemical compound as in claim 1, 2, 3, 4 or 5 wherein a plurality of chemical compounds are screened simultaneously using the same culture dish.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10874

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/18, 1/02; C12N 5/06, 5/02, 5/00, 1/00.
US CL :435/29, 32, 240.2, 240.23, 240.243.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 32, 240.2, 240.23, 240.243.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: search terms: hetatotoxicity, hepatic, liver, primary hepatic cell culture, choleostatic potential

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IN VITRO, Volume 17, No. 11, issued 11 November 1981, Inmon et al, "Development of a Toxicity Test System Using Primary Rat Liver Cells", pages 1004-1010, see entire document.	1-13
Y	US,A, 4,914,032 (Kuri-Harcuch et al) 03 April 1990, see entire document.	1-13
A	US,A, 5,032,508 (Naughton et al) 16 July 1991.	1-13
A	Cancer Research, Volume 36, issued May 1976, Carruthers et al, "Detection of Liver-bound Metabolites of Azocarcinogens by the Use of Anti-Hapten Antibodies", pages 1568-1571.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
10 JANUARY 1994	07 FEB 1994
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10874

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cancer Research, Volume 36, issued May 1976, Merski et al, "Effects of Andriamycin on Ultrastructure of Nucleoli in the Heart and Liver Cells of the Rat", pages 1580-1584.	1-13